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Journal of Chromatography A 817 (1998) 187–193

JOURNAL OF CHROMATOGRAPHY A

Determination of the minor whey protein bovine lactoferrin in cheese whey concentrates with capillary electrophoresis

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Abstract

In our present work we present the determination of bovine lactoferrin in whey concentrates as they are typically produced by milk and cheese industry after production of cheese. Due to the high total protein content the analysis of whey concentrate samples is difficult and even not possible by using capillary zone electrophoresis with UV detection. To enhance the detection sensitivity we applied a more promising approach by using affinity interactions in combination with laser-induced fluorescence detection. By mixing fluoresceine isothiocyanate (FITC)-conjugated polyanionic lipopolysaccharide with the mostly positively charged lactoferrin we found a significant migration time shift which is clearly dependent on the concentration of the added protein. In the second approach we developed an immunoassay using FITC-conjugated specific antibody against bovine lactoferrin. The results of the immunoassay measurements were compared with data obtained by standard enzyme-linked immunosorbent assay analysis.

Keywords: Whey; Affinity capillary electrophoresis; Immunoassays; Proteins; Lactoferrin

1. Introduction

Whey is a by-product from the cheese manufacturing process which is often used to produce whey protein concentrates powders for food applications. Due to the enormous volume the whey is almost concentrated about 5-times with evaporators before transportation to the whey-refining industry. Beside the major whey proteins [α-lactalbumin, β-lactoglobulin, bovine serum albumin (BSA) and bovine IgG] minor whey proteins are present such as lactoperoxidase, lacto(trans)ferrin and other minor compounds [1]. The glycoprotein lactoferrin (LF) belongs to the transferrin protein family with 3-fold stronger binding properties to iron than transferrin [2]. Although the protein was isolated over 35 years ago its function is still not totally elucidated. Beside the well known biological functions as an antimicrobial and antiviral agent the protein shows immunomodulatory functions in the host defence system [3]. Recently published results indicated that LF is also particular involved in Alzheimer's disease and could serve as human immunodeficiency virus (HIV) prevention clinical agent [4–9]. In human milk and external secretions it is one of the major proteins with concentrations between 1000 μg/ml in mature milk to 7000 μg/ml in colostrum [2] instead of only minor amounts in bovine milk ranging from 20–200 μg/ml [10]. Due to the basic isoelectric point (8.0–9.5) and the almost positive charge, bovine LF (bLF) can induce interactions with other

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0021-9673/98/$19.00
PII: S0021-9673(98)00445-2

whey and milk proteins like β-lactoglobulin and caseins [11].

The determination of bLF in whey is difficult because of the high concentrations of the major whey proteins and undefined lipid ingredients. The total protein content in sweet cheese whey yielded approximately 7–8 mg/ml resulting in a 70-fold lower protein content for the minor proteins. Analytical techniques for the determination of LF are reported using high-performance liquid chromatography (HPLC) [12] or fast protein liquid chromatography (FPLC) combined with ion-exchange chromatography [13]. The application of affinity chromatography for the isolation of LF has also been reported by several groups using heparin, Cibacron blue [14], antibodies [15], metal chelating compounds [16] or DNA [17]. The analysis of human major milk proteins including detection of human lactoferrin has been investigated with capillary zone electrophoresis (CZE), but the determination of bLF was not shown up tomorrow [18]. Recently, the affinity interaction of heparin and LF from different sources could be detected with affinity capillary electrophoresis (ACE) [19]. Nevertheless, CE analysis of bLF in whey concentrates as by-products of cheese manufacturing is very difficult to achieve as reported by our group very recently [20]. A more promising approach that we report here is the use of affinity interactions in combination with laser-induced fluorescence (LIF) detection. Two different approaches were made by using either the fluoresceine isothiocyanate (FITC)-conjugated polyanionic lipopolysaccharide (LPS) or FITC anti-bLF.

2. Experimental

2.1. Apparatus

All experiments were carried out on a Beckman P/ACE 2100 instrument (Beckman, Palo Alto, CA, USA) with LIF detection at 488 nm excitation and 520 nm emission wavelength using an argon ion laser with 2 mW power. System Gold software, version 8.10 (Beckman) and an IBM PS/1 personal computer were used for data collection, data analysis and system control. Fused-silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA) with an inner diameter of 50 μm. The total capillary length was 27 cm unless otherwise cited, i.e., 20 cm from the capillary inlet.

2.2. Reagents

All chemicals were purchased in the highest available purity grade. Sodium hydroxide, sodium sulphate, Na_2HPO_4 (12 H_2O), NaH_2PO_4 (12 H_2O) and boric acid were purchased from Fluka (Deisenhofen, Germany). Whey samples were a gift of BioLac (Harbarnsen, Germany), bovine lactoferrin (99.5% purity, lot 970728) was a gift of Morinaga Milk Industries (Tokyo, Japan). Polyclonal FITC-conjugated bLF antibodies (lot A10-126F-1) were purchased from Bethyl Labs. (Montgomery, TX, USA), FITC-LPS (serotype 026:B6, lot 74H4037) from Sigma (St. Louis, MO, USA).

2.3. Procedures

2.3.1. Capillaries

Before using the capillaries first time, they were etched with 1 *M* NaOH for 15 min followed by 5 min rinsing with deionized water and 5 min with electrophoresis buffer. After every run the capillaries were rinsed 5 min with 0.05 *M* NaOH and buffer. Sample injection was performed by applying 3.5 kPa pressure (0.5 p.s.i.) for 5 s (unless otherwise cited) to the sample vial placed at the anionic end of the capillary (1 p.s.i.=6894.76 Pa). The controlled temperature was 25.0±0.1°C (unless otherwise cited) during all experiments. The pH of the electrophoresis buffer (see titles of electropherograms) were adjusted with 1 *M* NaOH to the required pH.

2.3.2. Interaction with LPS (sero type026:B6)

For determination of the interaction of FITC-conjugated LPS and bLF or whey concentrate samples a solution of 100 μg/ml FITC-LPS in 100 m*M* phosphate buffer, pH 7.4 was incubated with 100 μg/ml bLF or 1:5 (v/v) deionized water diluted whey samples at room temperature for 15 min. All determinations were reproduced three times with new samples to eliminate errors of manual handling.

2.3.3. Immunoreaction

Antibody and substrates were dissolved in 100

m*M* phosphate buffer, pH 7.4. To perform the immunoreactions between FITC anti-bLF and antigenic samples equal volumes of 50 μl each were mixed in microvials and incubated at 37°C for 15 min using a thermomixer (thermomixer compact, Eppendorf-Netheler-Hinz, Hamburg, Germany). The FITC anti-bLF concentration for calibration and determination of the cross reactivity was 200 μg/ml, resulting in a total concentration of 100 μg/ml FITC anti-bLF after addition of the antigenic sample. bLF concentration for calibration was 0–150 μg/ml after mixture of the samples. Prior to the immunoreaction whey concentrate samples were diluted with deionized water 1:10 (v/v). For determination of cross reactivity to other whey proteins a concentration of 1000 μg/ml of bovine IgG, BSA, transferrin, β-lactoglobulin and α-lactalbumin in 100 m*M* phosphate, pH 7.4 buffer was used. After incubation samples were injected onto the capillary electrophoresis (CE) system. All determinations were reproduced three times with new samples to eliminate errors of manual handling.

2.3.4. Whey samples

For CZE and ACE analysis no pre-treatment of the provided whey concentrate samples was required. The samples were diluted to needed concentrations with deionized water as described above.

2.4. Enzyme-linked immunosorbent assay (ELISA)

ELISA measurements were carried out according to the user manual of the bLF ELISA kit (batch LF97H25) from BioX Diagnostics (Brussels, Belgium).

3. Results and discussion

According to the literature [18] the separation of major bovine milk proteins was reproduced with whey concentrate samples and compared to bLF dissolved in sample buffer under identical conditions (Fig. 1). The bLF electropherogram at 200 μg/ml concentration (lower panel) showed five peaks with low UV absorption at 14.5–16.1 min, indicating the separation of several glycoforms of bLF using borate buffer with the addition of sodium sulphate for

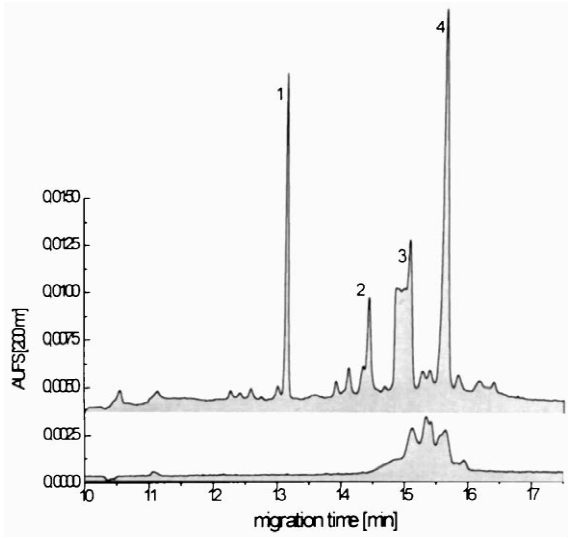

Fig. 1. CE of 200 μg/ml bLF (lower panel) and whey concentrate sample (upper panel). CE buffer: 100 m*M* borate, pH 8.2 and 30 m*M* Na_2SO_4. Conditions: applied voltage of 10 kV; temperature 25°C; 57 cm×50 μm fused-silica capillary; UV detection at 200 nm. 1=α-Lactalbumin, 2=BSA, 3=β-lactoglobulin B, 4=β-lactoglobulin A.

reduction of protein–wall interactions. The whey showed several peaks at the bLF migration time, one very insensitive belonging to β-lactoglobulin A (peak 4, upper panel), which comigrates with bLF. Under these conditions separation and quantitative analysis of bLF in whey is not possible. Additionally, investigation of other suitable buffers for CE analysis including micellar buffer systems did not yield sufficient results for the bLF determination in whey [20]: after 1:5 (v/v) dilution of the whey the bLF concentration is in the range between 20–200 μg/ml, which achieved low UV absorption signals. In the complex sample a baseline separation of bLF from other whey compounds is still not possible. To achieve more detection sensitivity we applied FITC-conjugated affinity ligands which can interact with bLF in whey samples.

3.1.1. FITC-LPS

The relationship between addition of bLF (2) and whey (3) to FITC-LPS and the observed migration time shift of the complex is shown in Fig. 2. The migration time shift between FITC-LPS (1) and a mixture of FITC-LPS and 100 μg/ml bLF (2) is

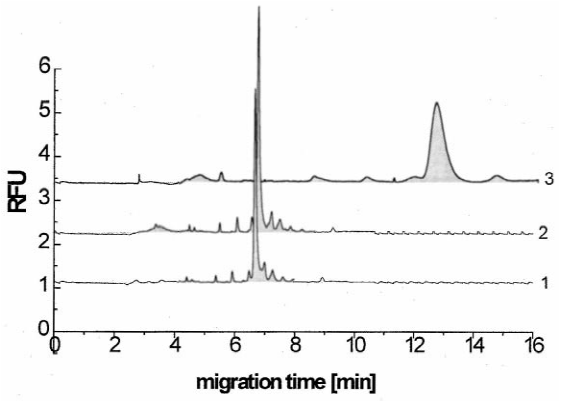

Fig. 2. CE of FITC-LPS and mixtures of FITC-LPS with bLF or whey. CE buffer: 100 m*M* borate, pH 10.6 and 30 m*M* Na_2SO_4, LIF detection at 488/520 nm, applied voltage of 10 kV; 27 cm×50 μm fused-silica capillary; temperature 20°C; concentration FITC-LPS 100 μg/ml; bLF 100 μg/ml; whey concentrate sample diluted 1:5 (v/v) with deionized water. 1=FITC-LPS, 2=FITC-LPS and bLF, 3=FITC-LPS and diluted whey sample.

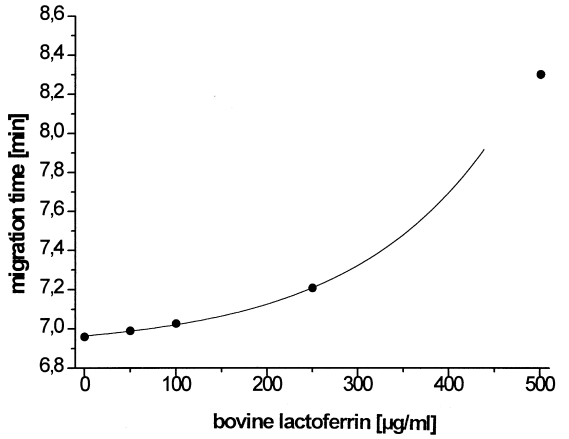

Fig. 3. Calibration fit for the determination of bLF with FITC-LPS using the migration time shift after adding different amounts of bLF. All other experimental conditions as in Fig. 2.

very low within the range of normal standard deviation (0.23 min).

By adding a whey sample to the FITC-LPS (3) a significant migration time shift of 6.1 min is achieved. Beside the shift in migration time the peak form is totally different compared to the FITC-LPS peaks obtained after adding bLF. The new peak form with increased width, area and decreased height indicates that there are more than one possible partners (e.g., β-lactoglobulin) in the whey sample present, which interact with the FITC-LPS. By variation of the bLF concentration we achieved an exponential relationship between the bLF concentration and the observed migration time shift of the FITC-LPS peak, as shown in Fig. 3. Extrapolation of the exponential calibration fit to the observed migration time shift in whey yielded a bLF concentration of 630±27 μg/ml. These results also indicates that other whey compounds interact with the FITC-LPS, because the bLF concentration in whey is 3- to 30-times too high. Apart of the increased migration time the peak area of the FITC-LPS is also increased in a similar way with a linear relationship by addition of bLF (data not shown).

3.1.2. FITC anti-bLF

A second approach was made by using polyclonal FITC-conjugated antibodies to perform an homogenous immunoassay. After adding the antibodies to different bLF concentrations we were not able to detect a new peak in the electropherogram corresponding to the formed immunocomplex. Even using reversed polarity did not achieve an immunocomplex peak. However, we found an exponential relationship between peak area decrease of the antibody and added amount of bLF (see Fig. 4) indicating, that both – antibody and immunocomplex – have similar electrophoretic mobility. The analysis of several whey concentrate samples were compared to data

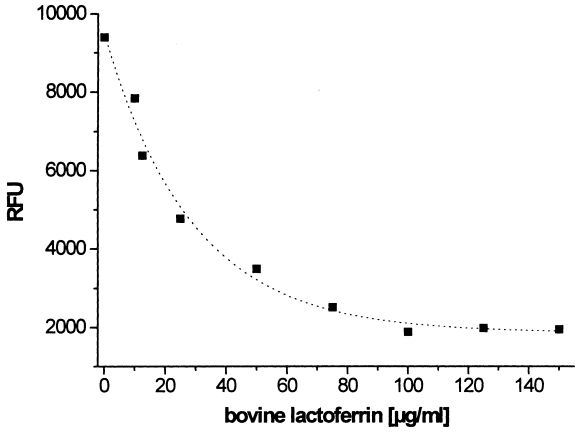

Fig. 4. Calibration fit for the determination of bLF with FITC anti-bLF. 200 μg/ml FITC anti-bLF, 0–150 μg/ml bLF; CE buffer: 100 m*M* phosphate, pH 7.4; temperature 25°C; 15 kV voltage applied; 27 cm×50 μm fused-silica capillary.

Table 1
Comparison of determined bLF in whey samples with ELISA and ACE

	Whey					
	1	2	3	4	5	6
ELISA (μg/ml)	116.24±2.44	124.70±3.20	117.91±3.32	113.87±3.39	126.49±2.00	99.50±7.54
ACE (μg/ml)	164.12±5.42	56.10±1.53	112.24±1.54	121.07±0.94	70.81±3.86	107.19±4.39
Δ*c* (μg/ml)	47.9	68.6	5.7	7.2	55.7	7.7

obtained with a commercial available ELISA kit (Table 1). The ELISA measurements of the six whey concentrate samples from different cheese manufacturers achieved bLF concentrations between 99.5 and 126.5 μg/ml with an average content of 116.5 μg/ml. The achieved data with ACE showed slightly different results with an average bLF content of 105.3 μg/ml. Although the average bLF content may induce similar results, the results are in detail different. Whey concentrate samples 3, 4 and 6 showed only minor deviations to the ELISA data. The differences between ELISA and ACE were found in the range 4.8–7.7% or 5.7–7.7 μg/ml, respectively. In samples 1, 2 and 5 we determined totally different concentrations with ACE and ELISA up to 69 μg/ml. A possible explanation could be the centrifugation and decantation of the whey samples prior to the ELISA measurements. During centrifugation an additional coprecipitation of bLF with whey compounds like β-lactoglobulin or casein is possible [15]. For ACE analysis we did not use the centrifugation step, because the ACE method should be very robust and rapid for on-line monitoring of whey concentrate samples during the industrial isolation process of bLF.

However, all determined bLF concentrations are lower than normally analysed in whey samples. The reason is still not clear, but could be caused by manufacturing of the different cheese arts due to the milk heterogeneity resulting in different protein contents in the whey. Additionally, the evaporation processes after the cheese production are not identical and caused also different protein amounts after the evaporation of 80% water.

To prove the reliability of the used FITC-conjugated anti-bLF we investigated the cross reactivity with other whey proteins (Fig. 5). The negative control in column 1 shows the peak area of a 1:1 dilution of FITC anti-bLF with the protein sample buffer used for solving the whey proteins in columns 2–6. As limit of cross reactivity we set up a relative deviation of 5% of the negative control peak area. The drawn horizontal lines corresponded to the 5% limit. The concentrations of the proteins we used were 1000 μg/ml for possible antigens and 110 μg/ml for the positive control with bLF.

The cross reactivity of transferrin with a structural homology to LF of about. 60% was lower than 5%, corresponding to 1.26 μg/ml bLF (see Table 2). The other tested antigens showed different cross reactivities between up to 9.56 μg/ml for BSA. For evaluation of cross reactivity present in whey the achieved value of the proteins are multiplied according to the concentration normally found in whey [10,21]. Transferrin, BSA and α-lactalbumin showed minor interactions between 0.13 and 3.8 μg/ml. The highest value was calculated for β-lactoglobulin with 15.59 μg/ml. Addition of all values in these experiments achieved a relative cross reactivity corre-

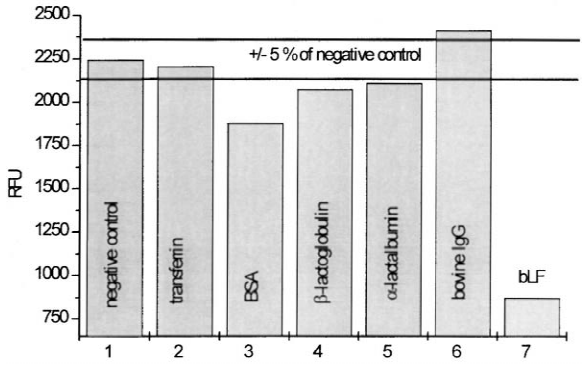

Fig. 5. Cross reactivity of FITC anti-bLF. 200 μg/ml FITC anti-bLF: negative control column: 100 m*M* phosphate buffer, pH 7.4; protein concentration columns 2–6: 1000 μg/ml in 100 m*M* phosphate buffer, pH 7.4; bLF: 110 μg/ml in 100 m*M* phosphate buffer, pH 7.4. CE conditions as in Fig. 4.

Table 2
Comparison of determined cross reactivities

Tested whey protein	Cross reactivity with ACE (μg/ml)	Factor	Cross reactivity in whey (μg/ml)
BSA	9.56±0.29	0.4	3.82±0.11
α-Lactalbumin	3.29±0.04	1.1	3.62±0.05
β-lactoglobulin	4.33±0.02	3.6	15.59±0.08
Transferrin	1.26±0.03	0.1	0.13±0.003
Total cross reactivity			23.16±0.24

sponding to approximately 23 μg/ml bLF present in whey. Additionally different conditions during cheese manufacturing and whey refining, especially precipitation and conglomeration reactions, caused different protein contents in the whey, which leads to high deviations between ELISA and ACE in samples 1, 2 and 5. To overcome these problems ACE experiments using a monoclonal antibody as in ELISA are now under investigation.

4. Conclusions

The application of FITC-conjugated LPS and FITC anti-bLF for determination of bLF in whey concentrates was shown. Concerning the interaction of FITC-LPS and bLF we found a significant migration time shift, depending on the added amount of bLF. Due to the polyanionic character of the LPS and possible interactions with other whey ingredients a determination of bLF in whey is not possible. The analysed whey samples showed migration time shift and increased peak area corresponding to a concentration of 630 μg/ml, which exceeds the normal bLF content about a factor of 3 to 30.

To achieve more reliable data from whey concentrates we used polyclonal FITC anti-bLF in a second approach. Even after reversed polarity of the electrodes we were not able to achieve a peak of the formed immunocomplex with bLF. However, we found a relationship between added amount of bLF and decreased peak area/heights of the FITC anti-bLF. After calibration we determined 56–164 μg/ml bLF in whey concentrates. The comparison with additional ELISA measurements showed, that the obtained data are sometimes in very good agreement with the ELISA. On one hand these concentration differences could be caused by a centrifugation and decantation step prior to ELISA analysis and a various loss of the whey proteins during cheese manufacturing and water evaporation. On the other hand the low specificity of the polyclonal antibody lead to a sensitivity loss by cross reaction with other whey proteins. Due to the refining process the protein content and the cross reactivity is dependent on several technical parameters. The tested cross reactivity with possible whey protein antigens showed that there are interactions with β-lactoglobulin, α-lactalbumin and BSA, which leads to relative cross reactivity of 23 μg/ml bLF. As a conclusion these problems will be eliminated by use of a monoclonal antibody raised against bLF of highest purity in hybridoma cells, which is still under investigation.

Acknowledgements

The authors would like to thank Ko Shiino from Morinaga Milk Industries (Stuttgart, Germany) for kindly providing us with bovine lactoferrin samples of highest purity. Additionally we thank the BioLac GmbH for the many whey concentrate samples. Special thanks to Dominic Petitjean from BioX Diagnostics (Brussels, Belgium) for the ELISA evaluation kit.

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